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Expression patterns of ROS responsive genes on boron-stressed canola (*Brassica napus* ssp. *oleifera* L.) following selenium treatment

Bor stresine maruz bırakılmış kanola bitkilerinde (*Brassica napus* ssp. *oleifera* L.) selenyum uygulaması sonrası ROS tepki genlerinin ifadelerinin belirlenmesi

M. Özge ÖZEN, Şeyda KAYA, Senem UYLAŞ, Durmuş ÇETİN, Esin ARI, M. Aydın AKBUDAK

Department of Agricultural Biotechnology, Akdeniz University, Antalya, Turkey

Corresponding author (*Sorumlu yazar*): M. A. Akbudak, e-mail (*e-posta*): akbudak@akdeniz.edu.tr
Author(s) e-mail (*Yazar(lar) e-posta*): melahatozgeozen@gmail.com, sydkya@gmail.com, senemuylas@gmail.com, durmuscetin42@gmail.com, esinari@akdeniz.edu.tr

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ABSTRACT

Though mitigating effect of selenium (Se) on various abiotic stresses is apparent, the knowledge on B-stress is very scant. Therefore, this work attempted to reveal its effects on B-stressed canola (*Brassica napus* ssp. *oleifera* L.) plants. In the present study, foliar gene expression and amounts of major antioxidant enzymes were investigated under different concentrations of individual (B or Se) and combined (B + Se) applications. The individual applications (per L) included 1.5 g B, 3 g B, 3 mg Se and 6 mg Se, while the combined applications were 1.5 g B+3 mg Se, 1.5 g B+6 mg Se, 3 g B+3 mg Se and 3 g B+6 mg Se. Under the given treatments, *APX*, *CAT*, *SOD*, *POD* and *GR* genes were mostly downregulated while some also showed upregulation such as *APX* (1.5 g B+6 mg Se and 3 g B+6 mg Se), *SOD* (1.5 g B+3 mg Se), *CAT* (3 g B, 3 mg Se and 3 g B+6 mg Se) and *POD* (1.5 g B). Assaying also implicated that beneficial effect of Se might be associated with the presence of stressor; otherwise, Se itself might induce the antioxidant mechanism as stressor. This work provides the results of individual and combined effects of B and Se applications on antioxidant gene expressions and protein activities in canola.

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ÖZ

Selenyumun (Se) çeşitli abiyotik stresler üzerindeki azaltıcı etkisi üzerine çalışmalar mevcut olmakla birlikte, B-stresi üzerindeki etkisi hakkında bilgi oldukça azdır. Bu nedenle, bu çalışmada B-stresine maruz bırakılmış kanola (*Brassica napus* ssp. *oleifera* L.) bitkileri üzerinde selenyumun etkilerinin ortaya konulması hedeflenmiştir. Ayrı ayrı (B veya Se) ve eş zamanlı (B + Se) uygulamaların belli başlı antioksidan enzimlerinin yapraklardaki gen ekspresyonu ve protein miktarları üzerine etkisi araştırılmıştır. Yapılan ayrı ayrı uygulamalar (litrede) 1.5 g B, 3 g B, 3 mg Se ve 6 mg Se'yi içerirken, eş zamanlı uygulamalar 1.5 g B + 3 mg Se, 1.5 g B + 6 mg Se, 3g B + 3 mg Se ve 3 g B + 6 mg Se içermektedir. Uygulamalar altında, APX, CAT, SOD, POD ve GR genlerinin çoğunlukla ifadeleri azalırken, APX (1.5 g B + 6 mg Se ve 3 g B + 6 mg Se), SOD (1.5 g B + 3 mg Se), CAT (3 g B, 3 mg Se ve 3 g B + 6 mg Se) ve POD (1.5 g B) gibi bazılarının ifadeleri ise artış göstermişlerdir. Bu çalışma Se' nin yararlı etkisinin ortamda herhangi bir stres etkeninin var olup-olmadığı ile ilişkili olabileceğini; aksi takdirde, Se' nin kendisinin doğrudan stres etkeni olarak antioksidan mekanizmayı indükleyebileceğini ortaya koymaktadır. Çalışmayla ayrıca, B ve Se uygulamalarının antioksidan gen ifadesi ve protein aktiviteleri üzerindeki eş zamanlı etkilerinin nasıl sonuçlandığı da gösterilmiştir.

1. Introduction

The essentiality of selenium (Se) has not been demonstrated in higher plants, but it is reported to be beneficial in only some of them (Filek et al. 2008; Pedrero et al. 2008; El-Ramady et al. 2016). Se exists in the nature in both inorganic (SeO_4^{2-}, SeO_3^{2-}, Se^{2-} and Se) and organic (SeCys and SeMet) forms (Sors et al. 2005; Bodnar et al. 2012), but it is taken up by plants mainly in the most prevalent/bioavailable form (selenate, (SeO_4^{2-})) via sulfate transporters. Due to its chemical similarity to sulfur, in

S-assimilatory pathway, it is reduced to selenite (SeO_3^{2-}) and then to selenide (Se^{2-}) and incorporated into amino acids as selenocysteine (SeCys), selenocystathionine (SeCysth) and selenomethionine (SeMet) (El-Ramady et al. 2016; Gupta and Gupta 2016). Selenium is reported to be involved in heavy metal uptake/transport inhibition, ROS and antioxidant regulation, photosynthetic system and cell membrane construction (Pilon-Smits 2015). Therefore, diverse beneficial effects of Se in plants from growth and development to stress tolerance are projected (Feng et al. 2013; Diao et al. 2014; Saidi et al. 2014; Hajiboland et al. 2015; Hawrylak-Nowak et al. 2015; Iqbal et al. 2015; Jiang et al. 2015; Naz et al. 2015; Qing et al. 2015; Tang et al. 2015). However, those beneficial effects have been mainly associated with the accumulation capacity of the plants since higher Se concentrations are toxic (El-Ramady et al. 2016; Gupta and Gupta 2016). It was given that major Se-hyperaccumulators in *Astragalus*, *Conopsis*, *Neptunia*, *Stanleya* and *Xylorhiza* genera could accumulate Se>1000 mg kg^{-1} of their dry weight. On the other hand, secondary-accumulators such as *Brassica juncea*, *B. napus*, *B. oleracea*, *Medicago sativa* and *Helianthus* spp. could accumulate up to 100-1000 mg kg^{-1}, while non-accumulators could take up less than 100 mg kg^{-1} Se of their dry weight (Galeas et al. 2007; Bodnar et al. 2012). Several studies also showed that optimal (usually low) concentrations of Se protects the plants from various abiotic stresses such as drought (Hasanuzzaman and Fujita 2011), cold (Chu et al. 2010), desiccation (Pukacka et al. 2011), temperature (Djanaguiraman et al. 2010), excess water (Wang 2011), UV (Yao et al. 2010) and heavy metals like As (Feng et al. 2009), Cd (Sun et al. 2010), Cr (Belokobylsky et al. 2004), Sb (Feng et al. 2011), Hg (Shanker et al. 1996), Al (Cartes et al. 2010) and Pb (Mroczek-Zdyrska and Wojcik 2012).

The mitigating effect of Se on many abiotic conditions has been reported from various plants, but the knowledge regarding its effects on boron (B) stress was very scarce. B is an essential plant micronutrient; still, its excessive or toxic concentrations cause significant reductions in crop yield and quality, especially in arid and semi-arid regions (Camacho-Cristobal et al. 2008; Landi et al. 2012; Ayvaz et al. 2013; Yoshinari and Takano 2017; Shireen et al. 2018). A globally important oilseed crop, *B. napus* (canola) also requires boron because it is crucial for male/female reproductive organs development and flower fertilization (Fletcher et al. 2016; Metwally et al. 2018).

Present work aimed to investigate the ameliorative effect of Se on B-stressed *B. napus* plants by evaluating the expression and protein activities of some antioxidant enzymes (APX, CAT, SOD, POD and GR), cellular lipid peroxidation levels and toxicity-derived morphological changes.

2. Materials and Methods

2.1. Plant material and growth conditions

Seeds of a commercial canola (*Brassica napus* ssp. *oleifera* L.) cultivar, DEO12-1471192 (Osterras Ag. Co., Antalya, Turkey), were used in the present study. Seeds were individually planted in 10-cm square-top pots containing 25% perlite and 75% peat. Plants were grown at 25°C and 50% humidity in a growth chamber under LD (140 µmol m^{-2} s^{-1}) with 16h-light/8h-dark regime. Plants were divided into nine groups, treatments of which are given below. Each plant were watered with 30 ml of ½ Hoagland solution every third day. On the 12th day of growth, plants were watered with 30 ml Hoagland solution containing boron (B) as boric acid (H_3BO_3) and/or selenium (Se) as sodium selenite (Na_2SeO_3). No boric acid or sodium selenite was added into Hoagland solution used for watering the control plants.

The groups 1-9 included the following treatments respectively; control, 1.5 g l^{-1} B, 3 g l^{-1} B, 3 mg l^{-1} Se, 6 mg l^{-1} Se, 1.5 g l^{-1} B + 3 mg l^{-1} Se, 1.5 g l^{-1} B + 6 mg l^{-1} Se, 3 g l^{-1} B + 3 mg l^{-1} Se and 3 g l^{-1} B + 6 mg l^{-1} Se.

Following 12 hrs of the treatments, leaves of the plants were harvested for RNA isolation and protein assays.

2.2. RNA isolation

RNA from leaves were isolated using RNA Plant Mini Kit (Qiagen, Cat No: 74904) according to the manufacturer's instructions. RNA samples were treated with RQ1 RNase-Free Dnase (Promega, USA). The intactness of RNA and DNA contamination were checked by gel electrophoresis. RNA amounts in the samples were determined with Qubit (Invitrogen, USA).

2.3. Gene expression analysis

RT-qPCR was carried out using Light Cycler 96 System (Roche). *SOD, POD, CAT, APX, GR* genes expressions were quantified in 10 ng RNA samples using Luna Universal One-Step RT-qPCR Kit (NEB, USA). The forward and reverse primer sequences for genes were obtained from Ali et al. (2015). As an endogenous control, serine/threonine-protein phosphatase (*PPR*) gene was used as reference (Yang et al. 2014). Gene expression was determined using $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). Average C_T values were obtained from six biological and three technical replicates for each gene.

2.4. Enzyme activity assays

0.5 g canola leaves were grounded using liquid nitrogen, and homogenized in 1 ml 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1% polyvinylpyrrolidine (PVP-40), 0.2% Triton X-100 and 0.1 mM Phenylmethylsulfonyl fluoride (PMSF). Samples were centrifuged in a refrigerated centrifuge at 14 000 rpm for 20 minutes. Supernatants were kept on ice for analyses. Protein contents of the supernatants were determined by Pierce Coomassie (Bradford) Protein Assay Kit (Bradford, 1976). Standard microplate protocol was followed according to the manufacturer's instructions.

To determine lipid peroxidation, MDA contents of the samples were assayed using a method modified from Ohkawa et al. (1979). Based on this modified protocol, 0.2 g leaf tissue grounded in liquid nitrogen was suspended in 1 ml 5% Trichloroacetic acid (TCA). The homogenate was transferred into a clean 1.5 ml microfuge tube (Eppendorf), and centrifuged at 12 000 rpm in room temperature. Equal amounts of lysate and freshly prepared 0.5% thiobarbituric asit (TBA) in 20% TCA were mixed, and then incubated at 100 °C for 25 min. The samples in the tubes were chilled on ice till room temperature, and then centrifuged at 10 000 rpm for 5 min. Absorbance of the supernatant was measured at 532 nm and also 600 nm wavelengths to clear off non-specificity due to turbidity. 0.5% TBA in 20% TCA was used as blank. MDA content was assayed using an absorbance coefficient of 155 mM^{-1} cm^{-1}.

APX (EC1.11.1.1) activity was determined by a method proposed by Murshed et al. (2008) after some modifications. Based on this modified method, 5 µl lysate and 185 µl solution

composed of 50 mM potassium phosphate buffer and 0.25 mM ascorbic acid were added into the each well of a UV transparent 96-well microplate (Costar, USA). APX reaction was started by adding 5 µl 200 mM H_2O_2 into each well. Ascorbate peroxidase was assayed at 25 °C from the decrease in absorbance at 290 nm (an absorbance coefficient of 2.8 mM^{-1} cm^{-1}) in every 49 seconds for 5 minutes. Spectrophotometric readings at the same conditions before adding H_2O_2 were used as reference.

SOD (EC1.15.1.1) activity was assayed using a method based on photochemical reduction ability of NBT by SOD. According to this method, 185 µl of analysis mix composed of 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L-metionin and 75 µM NBT was distributed into each well, and 10 µl total protein of samples was added into the wells. At last, reactions were initiated by adding 5 µl riboflavin (1 mM) into each well. Reactions were mixed by pipetting, and kept under 5000 lux light at 25 °C for 15 min. Absorbance values were determined at 560 nm 3 times using microplate spectrometer. The reaction mixes kept in dark was used as control, and an analysis mix kept in light was used blank. One unit of SOD activity (U) was defined as an enzyme amount required 50% inhibition of NBT by photoreduction activity of the enzyme (Yildirim and Kaya 2017).

POD activity (EC1.11.1.7) was calculated by absorbance increase of guaicol oxidation at 470 nm in 1 min. 50 µl of total protein were added into a cuvette, and the reaction was started by adding 1 ml reaction mixture of 100 mM phosphate buffer (pH 7.0), 0.2% guaiacol, 0.57% H_2O_2. One unit of POD activity (U) was defined as the decomposed H_2O_2 amount resulting in 0.01 absorbance increase per minute (Chen and Zhang 2015).

3. Results and Discussion

Boron is an essential micronutrient for healthy plant growth and development. However, its excessive or toxic concentrations in agricultural areas or in irrigation water are serious constraints for agricultural production by limiting crop yield and quality (Yoshinari and Takano 2017; Shireen et al. 2018). As in most abiotic stresses, B-toxicity also induces formation of reactive oxygen species (ROS) (Foyer and Shigeoka 2011). As a response to B-toxicity, plants use different enzymatic and non-enzymatic mechanisms to ameliorate the effect of this stressor. APX, CAT, SOD, POD and GR, which were studied in the present work, are among the most crucial antioxidant enzymes which synergistically function in scavenging of reactive oxygen species (Das and Roychoudhury 2014). In many studies, it was shown that their scavenging roles were significantly increased by Se supplementation, low concentrations of which have ameliorative effects on various abiotic stresses including drought, cold, desiccation, temperature, water, UV and heavy metal (Feng et al. 2013; El-Ramady et al. 2016). Still, only few studies have been available about B-stressed plants under mitigating Se application. Considering the literature scarcity in this scope, the present work attempted to investigate the ameliorative effect of Se on B-stress in *B. napus* plants.

To this end, having raised in growth chamber for 12 days, *B. napus* ssp. *oleifera* (canola) plants were treated with B and/or Se applications for 12 hours, and harvested for foliar gene expression (*APX*, *CAT*, *SOD*, *POD* and *GR*), protein levels (APX, SOD and POD) and cellular MDA concentrations. The expression profiles of each antioxidant gene were investigated under individual (B or Se) or combined (B + Se) applications. Individual applications (per L) included 1.5 g B, 3 g B, 3 mg Se and 6 mg Se while combined applications had 1.5 g B + 3 mg Se, 1.5 g B + 6 mg Se, 3 g B + 3 mg Se and 3 g B + 6 mg Se. The expressions of the antioxidant genes were quantified using previously designed primers by Ali et al. (2015). Reported as one of the most stably expressed genes in *B. napus* (Yang et al. 2014), *PPR* was used as an endogenous control in RT-qPCR.

In addition, morphological changes in the plants were also observed having insights about the toxicity symptoms (Fig. 1). At early stages, the symptoms of B toxicity are barely distinguishable. However, a direct relationship is present between foliar B content and severity of toxicity. Therefore, diagnosis for B-toxicity is mainly conducted in plant leaves (Reid 2013; Princi et al. 2015). Leaf burn and necrosis are commonly used for assessing B-toxicity (Roessner et al. 2006; Sutton et al. 2007; Reid and Fitzpatrick 2009; Reid 2013; Princi et al. 2015). In this regard, the observed morphological changes in the present study (Fig. 1) were similar to the B toxicity symptoms in the literature such as drying leaf tips/edges, leaf burn or leaf cupping. Especially, 3 g l^{-1} B application clearly caused one leaf cupped downwards, and drying leaf tips/edges were also among the most observed other symptoms.

Under stress conditions, one of the major changes occurring in plants at molecular level is up- or down- regulation of the antioxidant enzyme genes. In Se-supplemented plants under various stresses, different combinations of antioxidant enzymes were mostly demonstrated to show increased activities, for example, APX, DHAR, MDHAR, GR, GST, GPX and CAT in rapeseed (Hasanuzzaman and Fujita 2011), APX, CAT and POD in rapeseed (Duan et al. 2014), POD and CAT in wheat (Yao et al. 2009), CAT, SOD and POX in sorghum (Djanaguiraman et al. 2010), POD, SOD and CAT in wheat seedlings (Yao et al. 2010), SOD, CAT, APX and GR in mungbean (Malik et al. 2012), SOD, APX and GR in white clover (Wang 2011), SOD, APX, GR and GPX in red seaweed (Kumar et al. 2012) and many others. Those studies also implicated that antioxidant enzyme responses to stress factors in the presence of Se were highly depended on stress levels (i), applied Se-concentrations (ii) and enzyme variants (iii). First, plants can cope with low stress levels; however, under severe cases increased enzyme activities against ROS are required. Second, antioxidant enzyme activities are increased by high Se application itself although low Se concentrations are beneficial. Third, enzyme variant or its cofactor type plays a crucial role how its regulation is changed.

In the present work, antioxidant genes *APX*, *CAT*, *SOD*, *POD* and *GR* were found to be mainly downregulated under given applications (Fig. 2). In *APX*, the combined 1.5 g B + 6 mg Se and 3 g B + 6 mg Se applications showed a notable 33% and 40% increase in the gene expression respectively. This could result from the ameliorative effect of 6 mg Se application under increased B-stress, because no individual B or Se application or other combined applications with 3 mg Se caused an increase in *APX* expression. Thus, 6 mg l^{-1} of Se may be an optimal concentration for *APX* induction. Besides, *GR* gene was noted to be downregulated in all applications. Except 1.5 g B + 3 mg Se treatment with slight increase, SOD also showed a decreased expression pattern under the given treatments. However, in both *GR* and *SOD* genes, the individual 3 mg and 6 mg Se applications resulted in downregulation of their expressions more than that of individual 1.5 g and 3 g B treatments. These reduced gene expressions under Se may be associated with decreased ROS activity; thereby, mitigating

Figure 1. Effects of 12 hrs individual (B or Se) and combined (B + Se) applications in *B. napus* plants. From left to right, individual applications (per L) include 1.5 g B, 3 g B, 3 mg Se and 6 mg Se, while combined applications include 1.5g B + 3mg Se, 1.5 g B + 6 mg Se, 3 g B + 3 mg Se and 3 g B + 6 mg Se.

effect of Se on stress. *CAT* gene was found to be upregulated in 3 g B, 3 mg Se and 3 g B + 6 mg Se applications while downregulated in the others. Notably, boron increase from 1.5 g to 3 g considerably upregulated the *CAT* expression (~43%), this may be associated with the oxidative stress exerted. Besides, the most differential expression was observed in *POD* gene; 1.5 g B treatment caused 21% increase in *POD* expression while 3 g B + 6 mg Se application caused 62% decrease in expression.

On the other hand, amounts of total foliar APX, SOD and POD proteins measured showed less correlation with their mRNA levels (Fig. 3). This was reasonable because the expression levels were quantified only for a single member of the given gene, but protein amounts were assayed as total including concentrations of all the expressed members under given condition. Many other studies also demonstrated that changes in mRNA and protein levels do not always well-correlated due to regulation controls at different stages (Vogel and Marcotte 2012; Silva and Vogel 2016). Herein, individual 6 mg l^{-1} Se application showed the highest APX enzyme activity followed by 3 mg l^{-1} Se (Fig. 3A). This implies that Se itself may exert stress in the absence of other stress factors. However, when applied with other stress factor/s as in the combined applications, Se substantially decreased APX activity reduction of which might be related with less ROS activity and thus positive effect of Se. Besides, for the SOD protein, individual 1.5 g l^{-1} B and 6 mg l^{-1} Se applications were noted with the highest enzyme activities respectively (Fig 3B). Similar results were also observed in the POD activity (Fig. 3C). All these indicating that alleviative effect of Se might be somehow associated with the presence of stress factor, and otherwise Se –itself– might trigger antioxidant mechanism as a stressor.

MDA concentrations were also measured in order to assess lipid peroxidation as a result of the given treatments. Lipid peroxidation stems from the extent of oxidative damage on cellular membranes exerted by a stress factor. MDA, a byproduct of lipid peroxidation, thereby correlates with the plant stress levels (Chen and Zhang 2015; Sheoran et al. 2015; Wang et al. 2015). In the present work, MDA amounts were only considerably increased in 1.5 g l^{-1} B application while its amounts in the other treatments were similar to or less than the control groups' (Fig. 3D).

Overall, the stress tolerance is achieved by a synergetic coordination of the different antioxidant enzymes, upregulation of which are mainly implicated in combating oxidative stress. However, the antioxidant enzymes in the present study were mostly downregulated, and more interestingly, the individual B applications were also observed not increasing the expression of

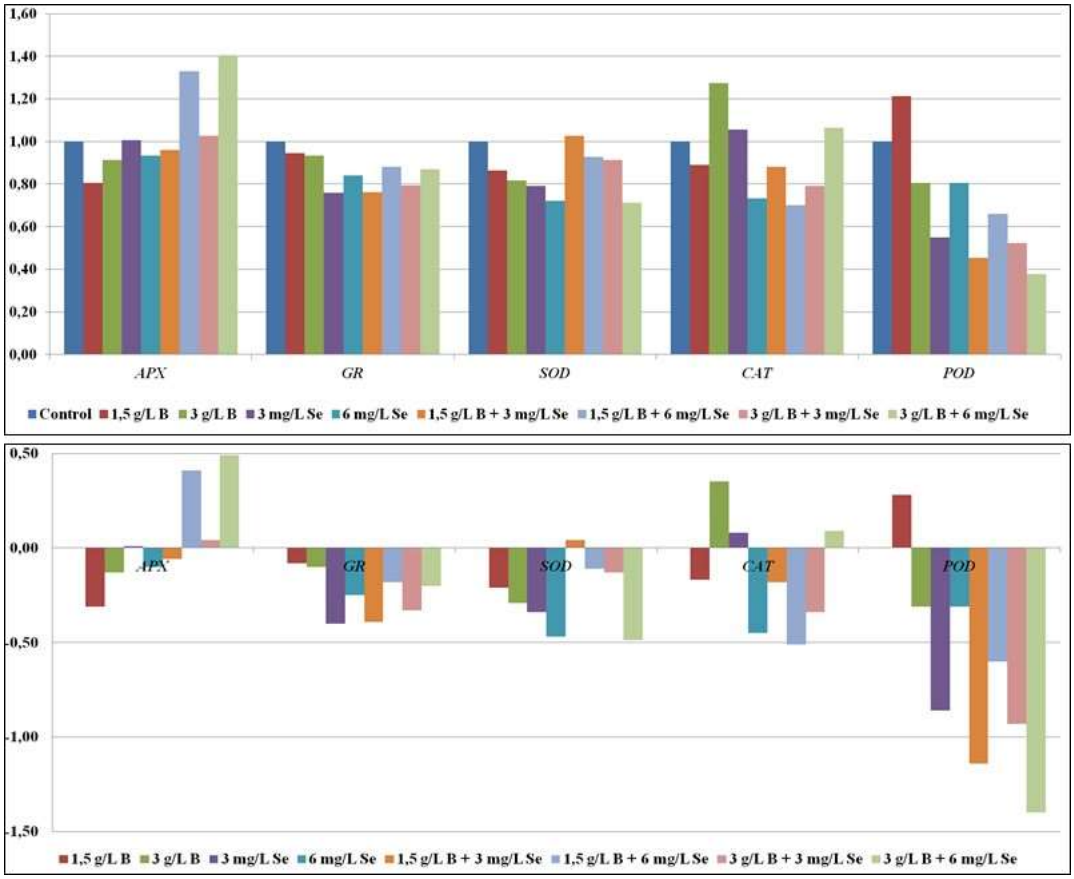

Figure 2. Foliar expression profiles of *APX*, *CAT*, *SOD*, *POD* and *GR* genes in 12 hrs individual (B or Se) and combined (B + Se) applications in *B. napus* plants. Quantification was performed by RT-qPCR. Data are represented as both fold change (above) and log2 (below) expression values for better presentation.

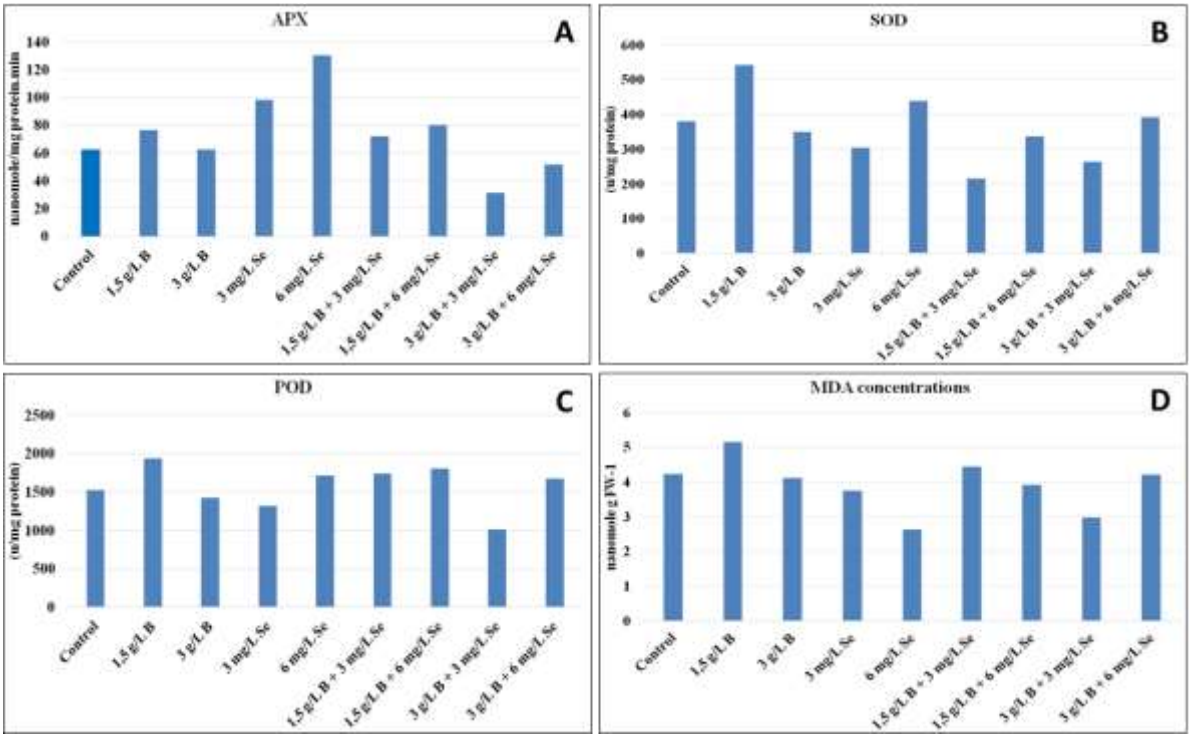

Figure 3. Total foliar APX (A), SOD (B), POD (C) and MDA (D) concentrations in 12 hrs individual (B or Se) and combined (B + Se) applications in *B. napus* plants.

the antioxidant genes in the most treatments. The downregulations in the gene expressions could be explained by that either the duration of the given treatments fall in short to induce sufficient ROS, or the ROS were already scavenged before the time point at which the expressions were measured. The latter explanation appears to be more relevant since the morphological observations also revealed that the symptoms were similar to B-toxicity in the some treatments. In case of translational point of view, the activities of the measured foliar antioxidant enzymes were less correlated with their mRNA levels.

4. Conclusion

Boron is an essential micronutrient, but its toxicity adversely affects plant productivity. Coping with it, plants are bestowed with various efficient antioxidant enzymes, synergetic coordination of which results in stress tolerance. In addition, optimal Se supplementation under stress conditions is also shown to further support the scavenging roles of these enzymes. The present study was therefore conducted to reveal the ameliorative effect of Se under B-stress. The antioxidant enzyme genes here were mainly downregulated, but the protein levels were less correlated with their mRNA levels. We think that the protein and the mRNA levels did not completely match because protein expression may take longer than 12 hours following stress induction. Different scenarios may also be proposed to explain it, but to understand the true reason behind requires further molecular studies under different concentrations of individual and combined stresses. In this regard, the present work provided a preliminary ground for future works, aiming to study ameliorative role of Se under B-stress plants.

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